

Identification of Factors Involved in Cell Integrity of Gram-negative Bacteria

Honors Research Thesis

Presented in partial fulfillment of the requirements for graduation *with honors research distinction* in Microbiology in the undergraduate colleges of The Ohio State University

by

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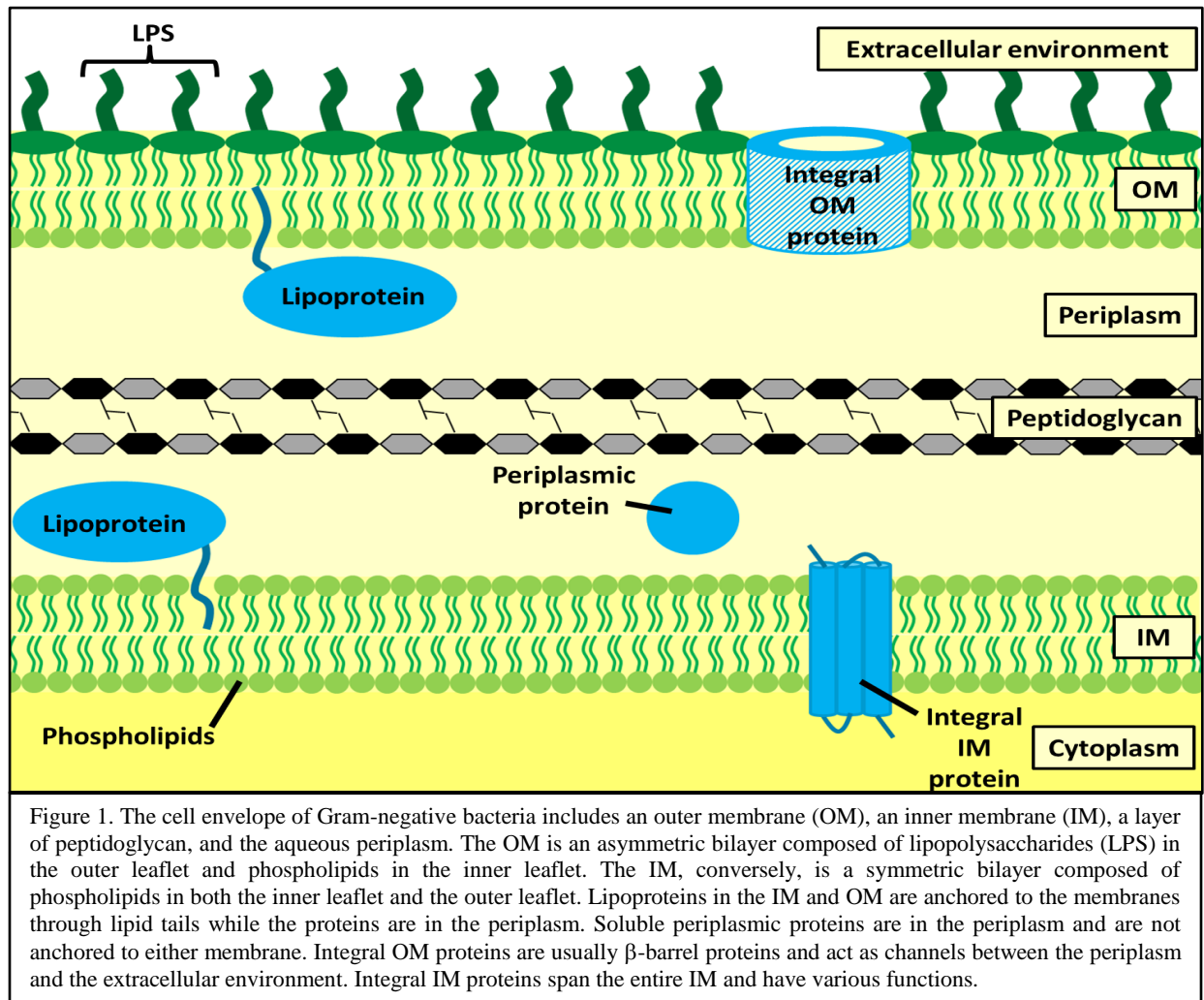
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## Abstract

The cell envelope of *Escherichia coli* and other Gram-negative bacteria is composed of an outer membrane and an inner membrane. Between the two membranes lies the aqueous periplasm which contains a layer of peptidoglycan. To maintain cell integrity, the synthesis and maintenance of the cell envelope must be carefully regulated. In order to identify factors involved in these processes, we developed a screen that identifies mutants with increased lysis (*ilm* mutants) among a population of transposon mutants. *ilm* mutants lyse more frequently than wild type because an aspect of their cellular integrity has been compromised. Our screen relies on the visualization of the release of cytoplasmic contents from colonies into the surrounding medium using a colorimetric reporter. Once these mutants are identified, the site of the transposon insertion can be determined, and the nature of the insertion and how it compromises cell integrity can be elucidated. Using our screen, we identified thirteen proteins important in maintaining cell integrity in *E. coli*. Twelve of these proteins have been characterized previously, while one protein, YciB, has not yet been characterized. Additional research is needed to fully understand the function of YciB in the maintenance of envelope integrity.

## Introduction

The cell envelope of *Escherichia coli* and other Gram-negative bacteria is composed of an outer membrane (OM) and an inner membrane (IM). The two membranes enclose an aqueous compartment known as the periplasm, which contains a layer of peptidoglycan (Fig. 1; Ruiz et al., 2006). The cell envelope provides a boundary between the environment and the cytoplasm of the cell, and this boundary is a main line of defense protecting the cell from stressful environments, such as environments that contain antibiotics, are acidic, or have low or high osmolarity. Because of its importance, defects in the cell envelope, such as increased permeability of the OM



or perforations in the peptidoglycan, can lead to lysis of the cell. These defects can arise if portions of the cell envelope are not properly synthesized or maintained. Therefore, the cell must have mechanisms that carefully regulate the proper synthesis and maintenance of the cell envelope. Additionally, the cell must have mechanisms that allow the cell envelope to acclimate and respond to environmental stressors so that the cell may survive in diverse and changing environments.

### Inner membrane

The IM surrounded the cytoplasm; therefore, proper biogenesis of the IM is crucial for cell integrity. The IM comprises two leaflets of phospholipids; the inner leaflet is adjacent to the cytoplasm, and the outer leaflet is adjacent to the periplasm. The IM contains proteins that are

either integral IM proteins or lipoproteins. Integral IM proteins are embedded in the two leaflets while lipoproteins are secured to the outer leaflet through a lipid anchor (Fig. 1; Ruiz et al., 2006). IM proteins are involved in numerous cellular functions. ATP synthase exists within the IM of Gram-negative bacteria and generates ATP using the electrochemical gradient across the IM (Futai et al., 1989). Other IM proteins sense stress and relay the information to the cytoplasm (Eguchi and Utsumi, 2008), shuttle proteins and cell envelope components across the IM from the cytoplasm (Ruiz et al., 2009), or transport nutrients to the cytoplasm (Bordignon et al., 2010). In *E. coli*, there are almost 1,000 different IM proteins (Misra et al., 2005), and these IM proteins must be properly synthesized and integrated into the IM in order to maintain cell integrity.

### **Outer membrane**

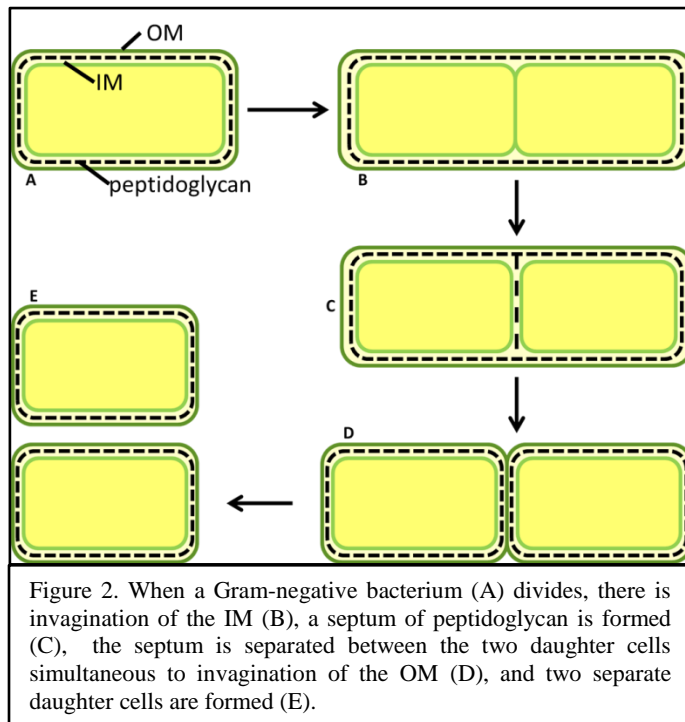
In *E. coli* the OM is a highly asymmetric lipid bilayer (Kamio and Nikaido, 1976). Its inner leaflet, which faces the periplasm, contains phospholipids, and its outer leaflet, which faces the exterior of the cell, contains lipopolysaccharides (LPS) (Fig. 1; Ruiz et al., 2006). LPS molecules are composed of lipid A, the core oligosaccharide, and the O polysaccharide (O antigen). Lipid A is the hydrophobic portion of LPS that anchors it to the OM. Attached to lipid A is the core oligosaccharide, which can be demarcated into the inner core and the outer core. Finally, the O polysaccharide is fixed to the outer core (Raetz and Whitfield, 2002).

OM proteins are, like IM proteins, either integral OM proteins or lipoproteins. Most integral OM proteins are  $\beta$ -barrels that span the OM.  $\beta$ -barrel proteins are cylinders formed by antiparallel  $\beta$  strands, and many of these form channels or porins linking the periplasm and the extracellular environment (Hagan et al., 2011). OM lipoproteins do not span the OM like integral OM proteins. Instead they are affixed to the inner leaflet through a lipid anchor (Fig. 1; Hagan et al., 2011).

The OM provides a very effective barrier against harmful molecules in the outside environment, such as antibiotics (Nikaido, 2003). The  $\beta$ -barrel channels allow the diffusion of only small hydrophilic molecules excluding larger molecules (Silhavy et al., 2010). In addition, LPS prevents the diffusion of many hydrophobic molecules which can diffuse easily across phospholipid bilayers, such as the IM (Nikaido, 2003). Increased OM permeability, therefore, allows the entry of antibiotics and other harmful molecules that can lead to death of the cell. Defects synthesis, transport, or assembly of LPS and OM proteins can increase OM permeability. Because the OM is an essential cellular component, these defects may also lead to cell lysis. Consequently, to maintain cell integrity, these processes must be carefully regulated.

### **Periplasm and peptidoglycan**

The periplasm is the aqueous compartment between the IM and the OM. It contains numerous soluble proteins (Fig. 1; Ruiz et al., 2006). Within the periplasm resides the peptidoglycan layer, a polymer mesh composed of long, cross-linked glycan strands containing the saccharides N-acetyl glucosamine and N-acetyl muramic acid (Vollmer and Bertsche, 2008). These rigid cross-linked strands form a sac around the cell, determine the cell's shape, and act as a barrier beyond which the cell cannot expand, thus contributing to the maintenance of cell shape and helping prevent lysis that can occur as a result of the high turgor pressure in the cytoplasm (Silhavy et al., 2010). Additionally, the peptidoglycan layer is covalently attached to the OM, securing the OM to the cell (Silhavy et al., 2010). During growth expansion of the peptidoglycan sac is necessary. For growth to occur, some of the existing peptidoglycan must be degraded, and new peptidoglycan strands must be synthesized and inserted into the preexisting polymer mesh (Vollmer and Bertsche, 2008). The degradation and synthesis of peptidoglycan must be carefully



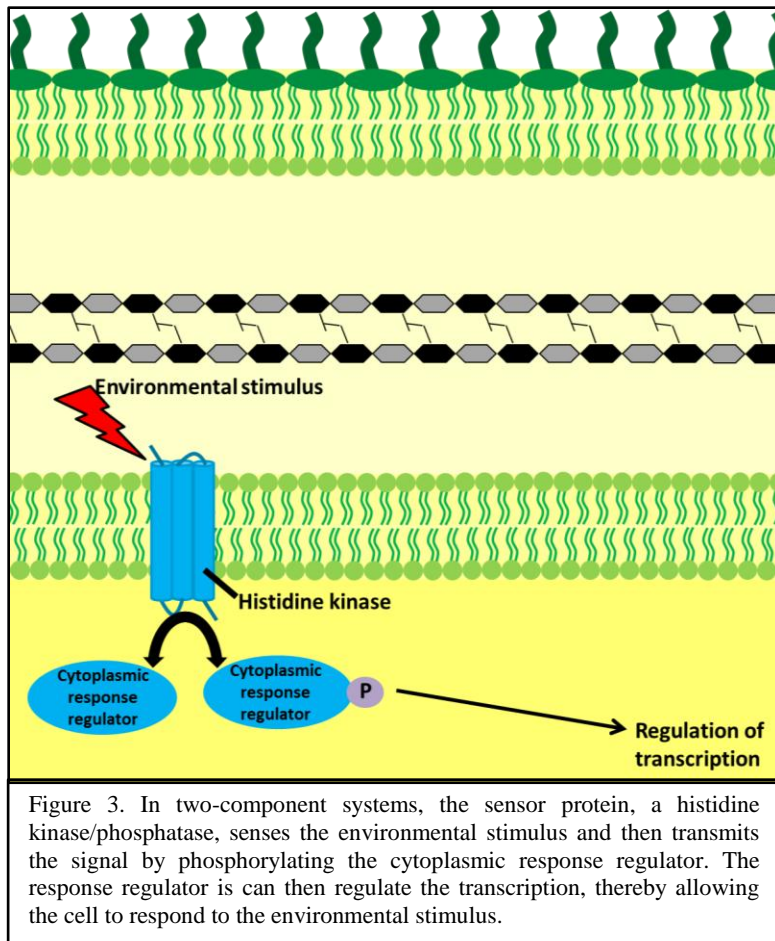
regulated and balanced because too much degradation or too little synthesis could lead to lysis of the cell.

### Cell septation and separation

Like other bacteria, *E. coli* propagates by binary fission (Fig. 2). The first step this division process involves the formation of a ring of the cytoplasmic protein FtsZ at the midcell, thereby marking the site of cell division.

This ring then recruits other proteins required for proper cell division, forming the divisome (Uehara and Bernhardt, 2011). These proteins at the divisome mediate construction of the septum: two layers of IM separated by a wall of peptidoglycan. After separation, the septum will become the new poles of the daughter cells (Uehara and Bernhardt, 2011). In order to form these poles, amidases degrade portions of the newly synthesized peptidoglycan, and the peptidoglycan is separated, thereby allowing each daughter cell to contain peptidoglycan at its newly formed pole (Uehara and Bernhardt, 2011). As this is occurring, the OM constricts to fully separate the two daughter cells (Uehara and Bernhardt, 2011).

Cell division is a process that must be carefully regulated. Like cell growth, it involves both synthesis and degradation of peptidoglycan and could potentially result in cell lysis. Inhibition of septum formation result in a filamentous phenotype: a long cell containing multiple chromosomes and lacking septa to sequester these chromosomes into separate cells (Harry et al., 2006). On the other hand, defects in degradation of septal peptidoglycan can prevent cells from



separating, resulting in long chains of cells; the septa in these cells are visible under the microscope. Both phenotypes can result in increased lysis.

### Regulation in the cell envelope

In order to maintain cell integrity, the cell envelope must be able to sense and acclimate to changing environmental conditions. Many protein sensors are located in the cell envelope.

These sensory proteins transduce

the signal to the cytoplasm, where regulation of transcription and translation can occur (Eguchi and Utsumi, 2008). Depending on the environmental cue, the cell will upregulate or downregulate the expression envelope components (Ades, 2008; Eguchi and Utsumi, 2008), and upregulate the expression of proteins that combat envelope stress (Ades, 2008). One environmental stress recognition and response process utilized by bacteria is the two-component system (Fig. 3). An integral IM protein, the histidine kinase/phosphatase, acts as the sensor protein. After sensing the environmental stimulus, the sensor transmits the signal by phosphorylating the cytoplasmic response regulator. The response regulator then controls the expression of genes whose products allow the cell to respond to the stressor (Eguchi and Utsumi,

2008). In the absence of stimulus, the sensor dephosphorylates the response regulator (Eguchi and Utsumi, 2008).

Regulatory pathways such as two-component systems have the potential to considerably affect the cell's permeability and the cell's ability to resolve malfunctions. Indeed, overexpression of certain stress responses can cause damage to the cell, while underexpression of stress responses in stressful environments can also lead to significant damage (Button et al., 2007; Hayden and Ades, 2008). Maintenance of sensory proteins and their cytoplasmic counterparts, therefore, is incredibly important for proper cell functioning and cell integrity.

This thesis focuses on a genetic screen designed to identify mutants that have compromised cell envelope integrity in *E. coli*. We expected to find mutants that are defective in the biogenesis of any of the envelope compartments as well as in the coordination of envelope components and the regulation of cell envelope stress responses.

## **Methods**

### **Media, growth conditions, and microscopy**

Luria-Bertani (LB) and M63 minimal media were prepared as described previously (Silhavy et al., 1984). All experiments were performed under aeration at 37 °C unless indicated otherwise. Cells were grown in LB broth for all experiments. Kanamycin (Gold Biotechnology) was used at a concentration of 25 µg/ml. Isopropyl-β-D-thiogalactopyranoside (IPTG, Gold Biotechnology) was used at a concentration of 0.2 mM in liquid media; 50 µl of 0.1 M IPTG and 50 µl of 20 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Gold Biotechnology) were spread on each plate, which contain about 30 ml of solid medium each. D-glucosamine (Sigma) was used at a concentration of 200 µg/ml in liquid media; 100 µl of 50 mg/ml D-glucosamine was spread on each plate. Bacteria were viewed using a Nikon Optiphot-2



microscope under 400X magnification, and pictures were obtained using a Canon DS126231 digital camera. Pictures of plates were obtained using a Casio EX-S500 digital camera. Pictures were modified in Microsoft Powerpoint 2010.

### **Bacterial strains and construction of transposon mutants**

MG1655 was the wild-type strain used for all experiments (Daniels et al., 1992). The population of transposon mutants was constructed using the EZ-Tn5 <KAN-2> Transposome (Epicentre) following the manufacturer's protocol. The location of the insertion of the EZ-Tn5 <KAN-2> Transposon was amplified using arbitrary PCR as described by O'Toole and Kolter (1998) using the primers ARB1 (5'-GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT-3') and EZTnExt (5'-TTGGTTGTAACACTGGCAGAGC-3') for the first reaction of arbitrary PCR and the primers ARB2 (5'-GGCCACGCGTCGACTAGTAC-3') and EZTnInt (5'-AAGCTCTCATCAACCGTGGCGG-3') for the second reaction. PCR products were sequenced by the Ohio State University Plant Microbe Genomics Facility. Alleles were transferred by P1 transduction. For selected genes, deletion alleles were obtained from the Keio Collection (Baba et al., 2006).

### **Screen for *ilm* mutants**

After electroporation of the EZ-Tn5 <KAN-2> Transposome, transposon mutants were selected on LB plates containing kanamycin, IPTG, and X-gal after overnight inoculation at 37 or 42 °C. The number of resulting colonies was counted, and colonies surrounded by a blue ring (*ilm* mutants) were isolated.

### **Identification of transposon insertion location in *ilm3-2***

The *envC* region in *ilm3-2* was amplified using by PCR using the primers 5envC3784761 (5'-ATGTTGTCGCTGATGGGTATGG-3') and 3envC3786171 (5'-CCAACAGAGCGGCAAATG-3') and visualized on an agarose gel. The amplified region is over 2 kb in length, whereas the gene *envC* is 1284 bp (Misra et al., 2005). The transposon is 1221 bp (Epicentre); therefore, the amplified region included both the gene *envC* and the transposon insertion, successfully confirming the transposons insertion location in *ilm3-2*.

### **Construction of pBADyibQ and pBADyciB**

Plasmids expressing *yibQ* and *yciB* under the control of the arabinose promoter were constructed by inserting the *yibQ* and the *yciB* open reading frames into the EcoRI and XbaI sites of pBAD18 (Guzman et al., 1995). The *yibQ* and *yciB* open reading frames were amplified by PCR using MG1655 genomic DNA as a template for both. The primers 5yibQEcoRI (5'-GCGAATTCACGAGGATAATGGAATGTTTCCAT TTCGTCGTAACGTTC; the EcoRI site is underlined) and 3yibQXbaI (5'-GCTCTAGACACCATCGCATTACGCGTAG; the XbaI site is underlined) were used to amplify *yibQ*, and the primers 5yciBEcoRI (5'-GCGAATTCTTTGATTACGGAATCGTA; the EcoRI site is underlined) and 3yciBXbaI (5'-GCTCTAGATTAGGATTTATCTTCCTG; the XbaI site is underlined) were used to amplify *yciB*.

### **Quantification of lysis**

$\beta$ -galactosidase assays were performed at room temperature using a variation of the microtiter assay described by Miller (1972). Cultures were grown overnight in 5ml LB broth at 37°C or at 42°C. Cells collected by centrifugation (Eppendorf Centrifuge 5418 at 14000 rpm), resuspended in 1 ml Z-buffer (Miller, 1972), and vortexed for 10 s after the addition of 2 drops of chloroform and 1 drop of 0.1% SDS. The supernatant was kept and made cell-free filtering it

through a membrane with a pore size of 0.4  $\mu$ l. 25  $\mu$ l of the cells and 150  $\mu$ l and 50  $\mu$ l of the supernatant were added to the microplate wells in triplicate (flat-bottom, Greiner Bio-One). The final volume was adjusted to 150  $\mu$ l with the addition of Z-buffer. The addition of 100  $\mu$ l of *o*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG; 5 mg/ml, Amresco) in Z-buffer without  $\beta$ -mercaptoethanol activated the reaction. The  $A_{420}$  was measured on Bio-Rad xMark microplate spectrophotometer, and 40 readings were taken at 15 s intervals. The maximum rate of  $\Delta A_{420}$  per minute was determined by the Bio-Rad Microplate Manager® software version 6.2. The formula used to calculate the release of  $\beta$ -galactosidase in culture is:

$$\frac{(\text{rate for supernatant of mutant grown at } X^{\circ}\text{C}) / (\text{rate for cells of mutant grown at } X^{\circ}\text{C})}{(\text{rate for supernatant of MG1655 grown at } X^{\circ}\text{C}) / (\text{rate for cells of MG1655 grown at } X^{\circ}\text{C})}$$

As described below, some mutants acquired suppressors easily. Cultures that developed suppressor mutations were not included in lysis indexes and standard deviations.

### **Western blots**

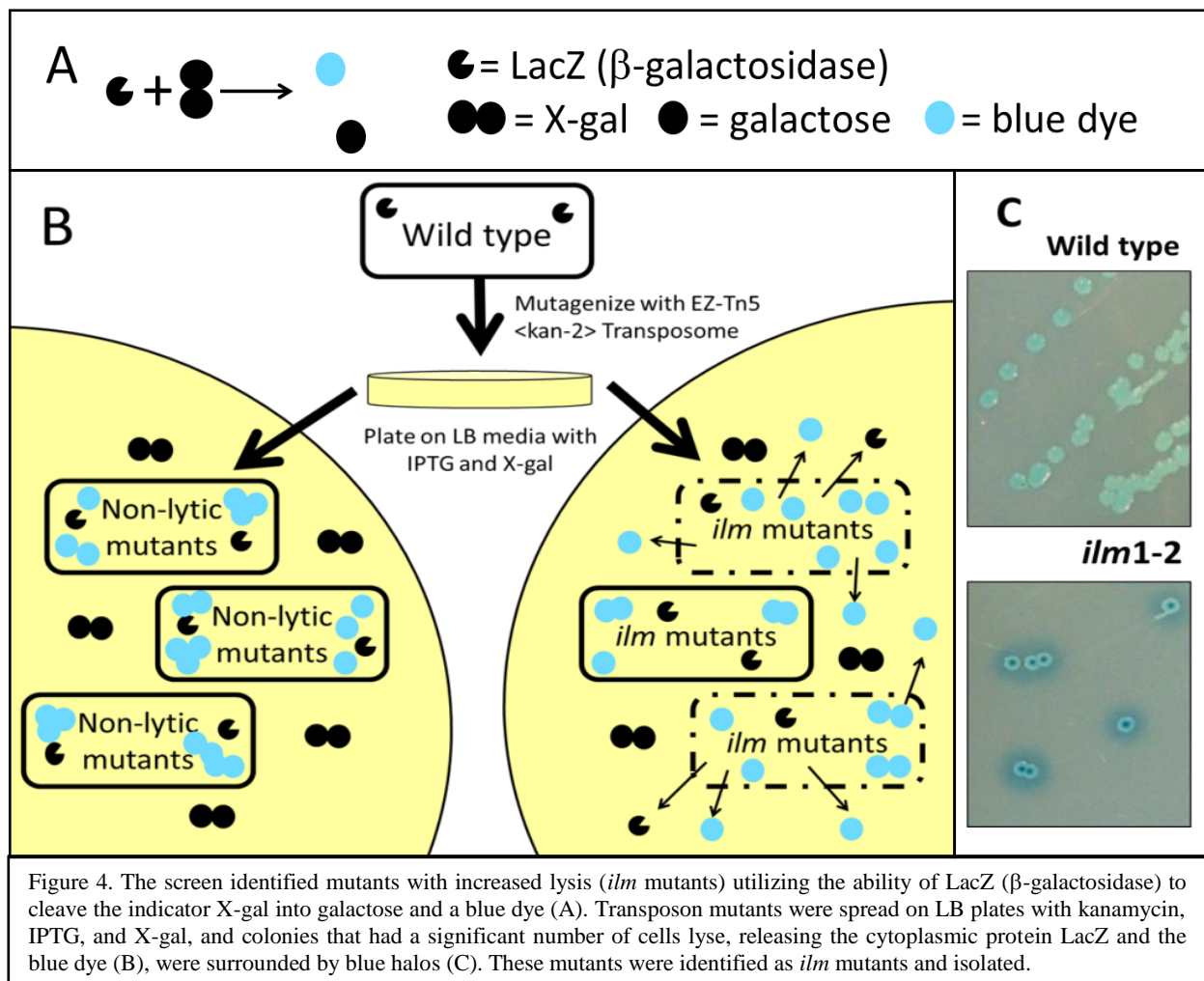
DegP levels were monitored by western blotting using a rabbit polyclonal anti-DegP antiserum as described previously (Button et al., 2007).

## **Results and Discussion**

### **A genetic screen to identify *ilm* mutants**

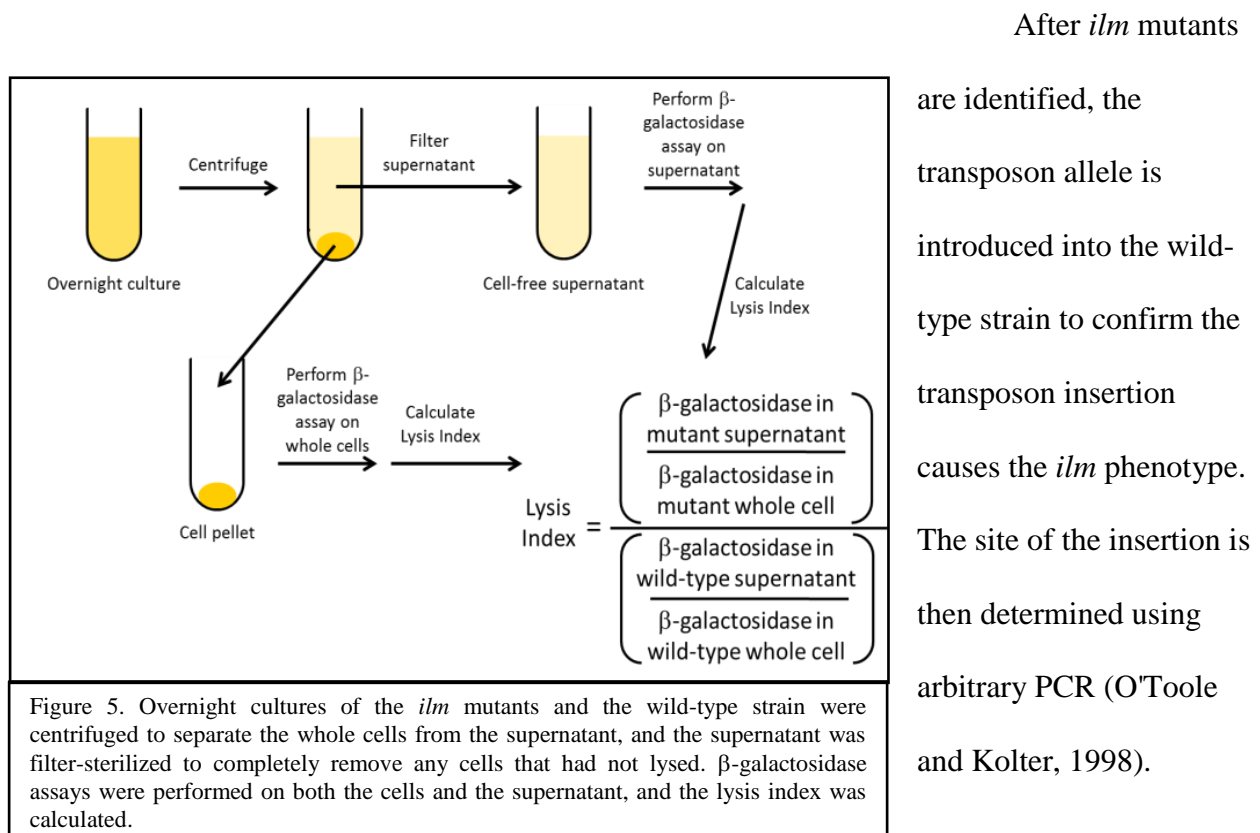
In order to identify factors contributing to cell integrity, we have developed a genetic screen that identifies mutants that have a phenotype of increased lysis (*ilm* mutants). In this screen *ilm* mutants are identified among a population of transposon mutants, using increased lysis as an indicator of compromised cell integrity. Specifically, the screen identifies colonies on solid media that contain an appreciable number of lysed cells that have released their cytoplasmic contents to the surrounding media.

The wild-type strain, MG1655, is capable of producing LacZ ( $\beta$ -galactosidase) (Misra et al., 2005), an enzyme that hydrolyzes lactose into glucose and galactose (Ghim et al., 2010). Isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) induces the production of this protein at the transcriptional level (Donovan et al., 1996). LacZ can also hydrolyze the indicator 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal) into galactose and a blue dye (Kiernan, 2007). Therefore, colonies that produce LacZ, such as the wild-type strain, are blue on media that contains IPTG and X-gal and the blue color is restricted to within the colony. If a fraction of cells in a colony lyse, they release their cytoplasmic contents, including the cytoplasmic protein LacZ. Additionally, any blue dye produced prior to lysis can diffuse into the surrounding media.



In this instance, the blue color is no longer contained within the colony, and the dye forms a blue halo around the colony. Because *ilm* mutants display an increased release of their cytoplasmic contents, the blue ring is visible around these colonies (Fig. 4). This allows for the visualization of *ilm* mutants.

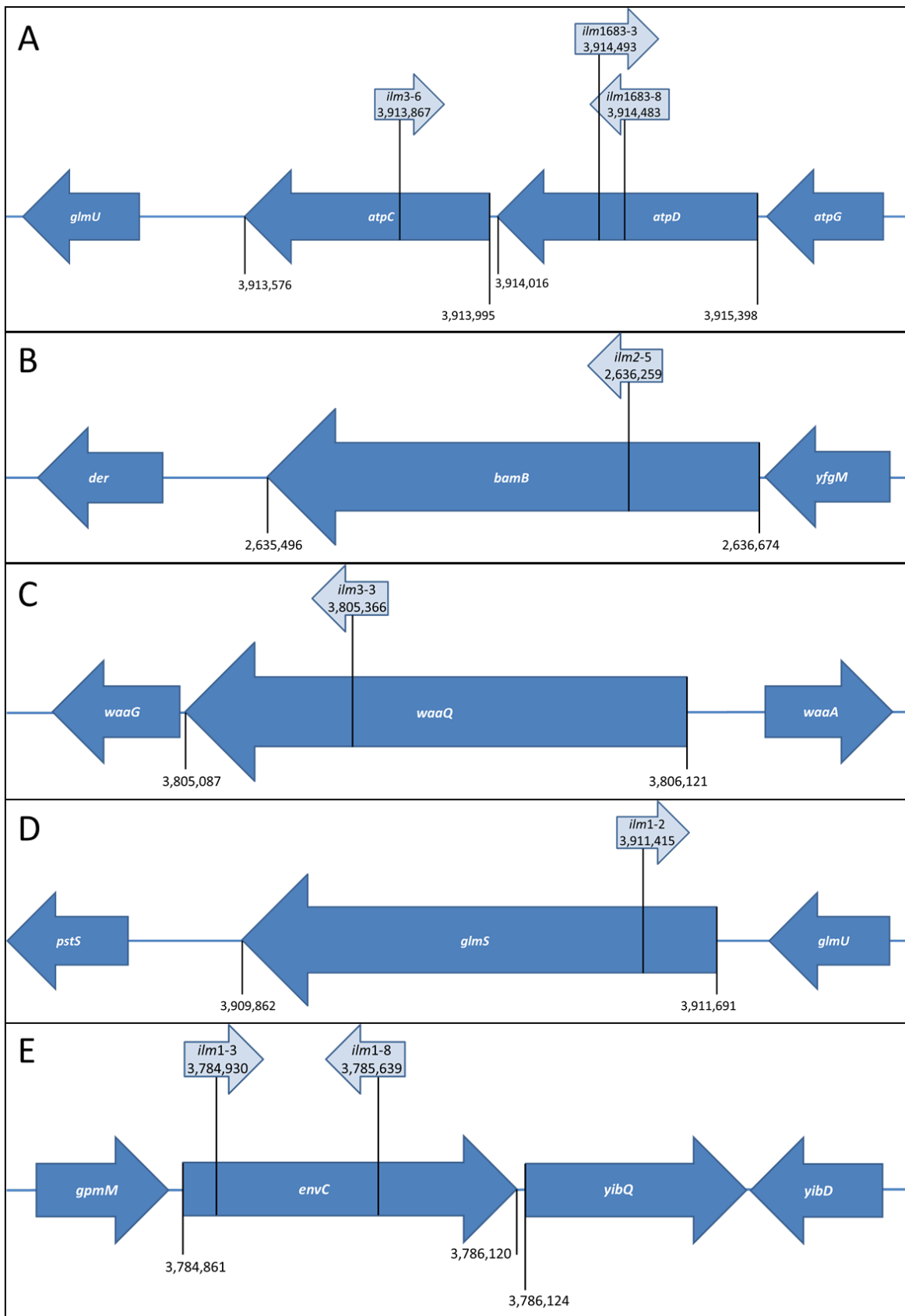
To quantify lysis, we also developed an assay base on the  $\beta$ -galactosidase assay described by Miller (Fig.5; 1972). Our assay allows us to compare the release of LacZ from the *ilm* mutants and wild type into the culture media to obtain a lysis index. The wild-type strain, therefore, has a lysis index of 1. A lysis index less than 1 indicates that the particular strain releases less LacZ and, therefore, less of its cytoplasmic contents in culture than wild type. Lysis indexes greater than 1 indicate that the *ilm* mutants release more LacZ and more of their cytoplasmic contents in culture than wild type. As described below, some mutants acquired suppressors easily.

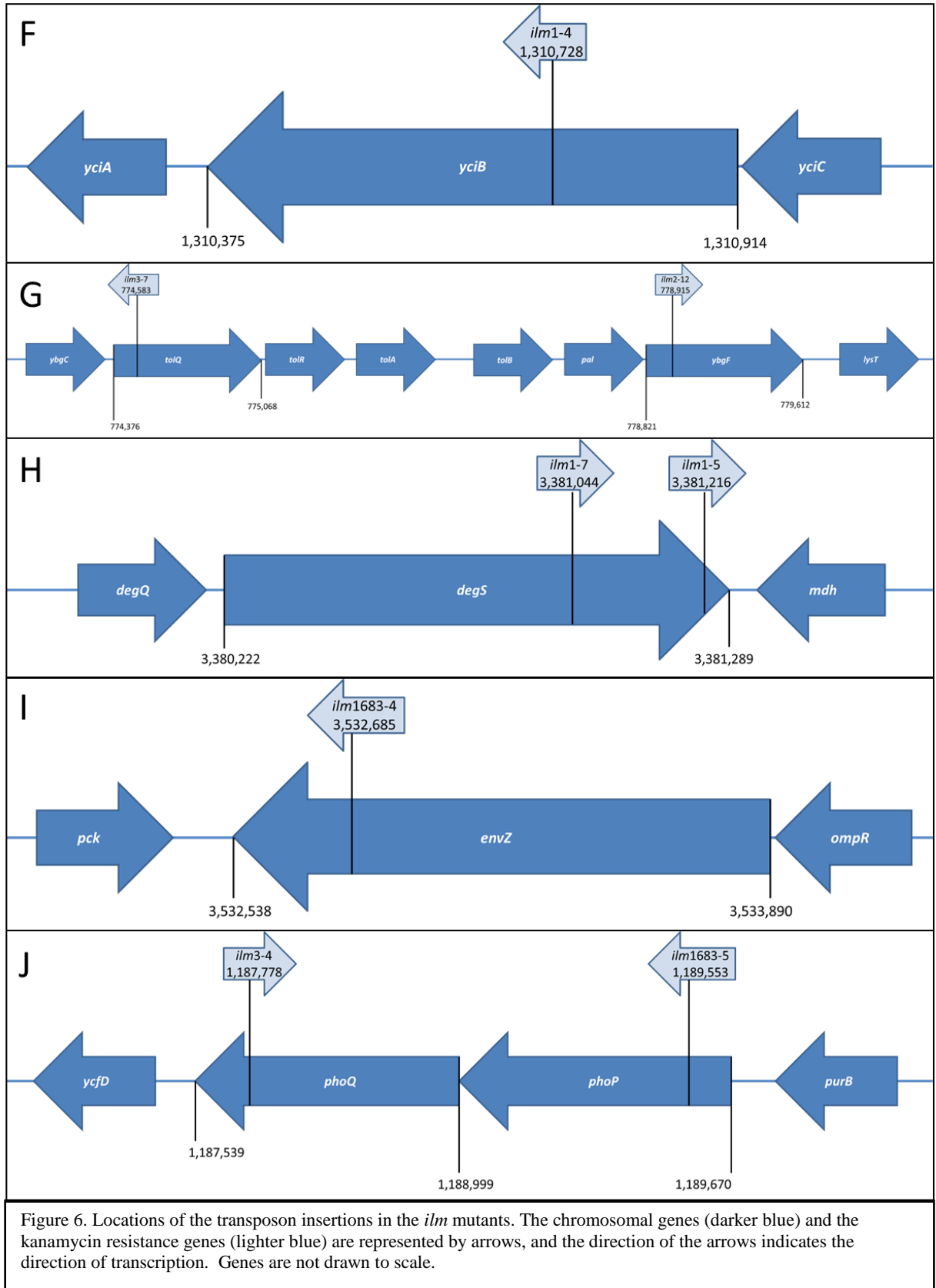


Phenotypes conferred by the *ilm* alleles are compared to their respective deletion alleles obtained from the Keio Collection (Baba et al., 2006) to determine if the insertion caused a loss of function or if more research is needed to determine if the insertion caused only a partial loss of function or a gain of function.

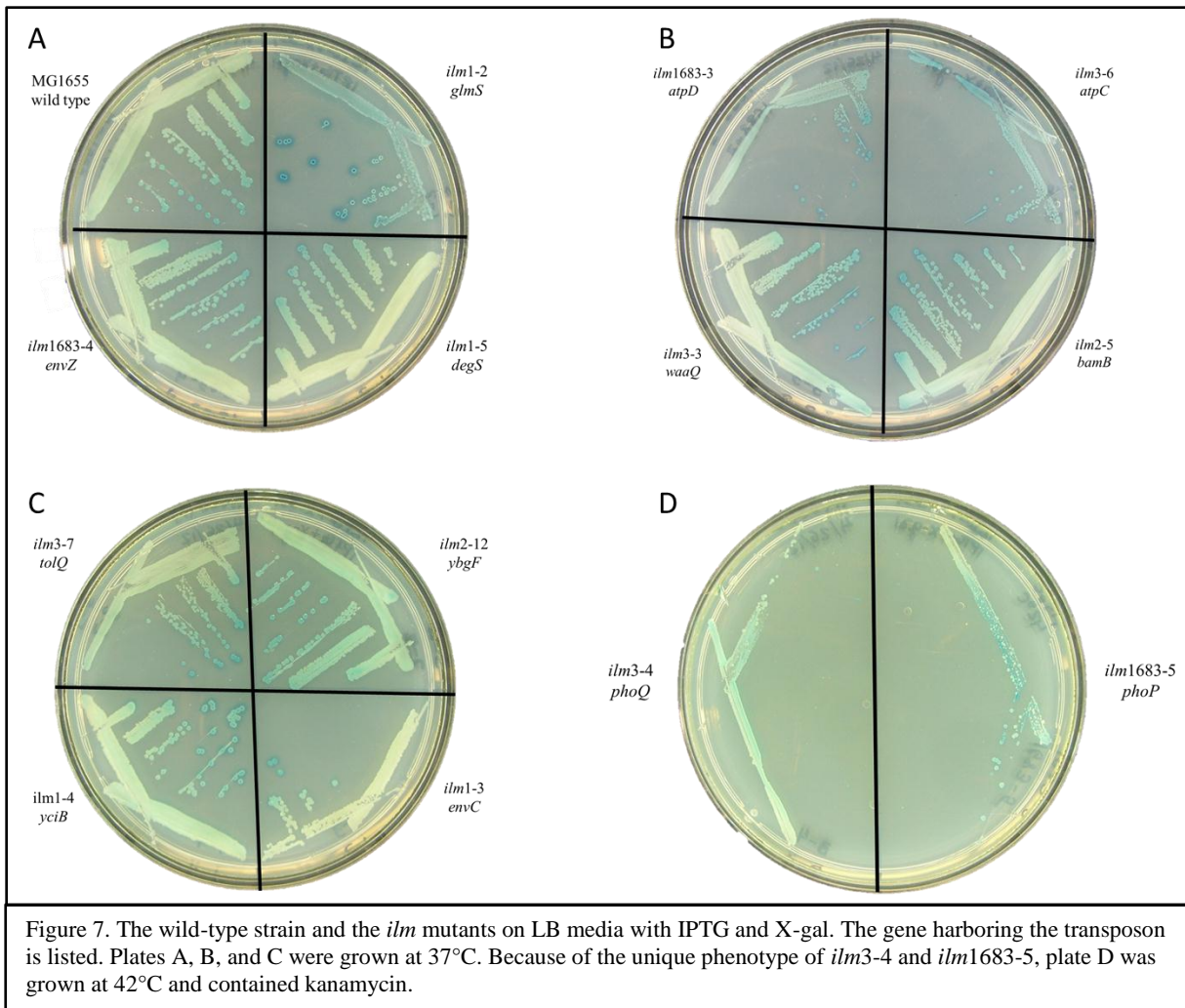
For simplicity, the results that follow have been organized by which cellular processes are defective in each *ilm* mutant.

<b>Table 1. Comparison of <i>ilm</i> phenotypes after growth of <i>ilm</i> mutants on solid media and in liquid media.</b>			
<b>Gene affected by transposon insertion</b>	<b>Increased lysis mutant</b>	<b>Increased lysis on solid media</b>	<b>Increased lysis in liquid media</b>
no insertion	MG1655 (wild type)	-	-
<i>atpC</i>	<i>ilm3-6</i>	+	+/-
<i>atpD</i>	<i>ilm1683-3, ilm1683-8</i>	+	+/-
<i>bamB</i>	<i>ilm2-5</i>	++	++
<i>waaQ</i>	<i>ilm3-3</i>	+/-	-
<i>glmS</i>	<i>ilm1-2, ilm1-6, ilm2-1, ilm2-2</i>	++++	++++
<i>envC</i>	<i>ilm1-3, ilm1-8, ilm3-2</i>	+++	++
<i>yciB</i>	<i>ilm1-4</i>	++	++
<i>tolQ</i>	<i>ilm3-7</i>	+++	++
<i>ybgF</i>	<i>ilm2-12</i>	+	-
<i>degS</i>	<i>ilm1-5, ilm1-7</i>	+++	++
<i>envZ</i>	<i>ilm1683-4</i>	+	+
<i>phoP</i>	<i>ilm1683-5</i>	+/-	+
<i>phoQ</i>	<i>ilm3-4</i>	+/-	+/-









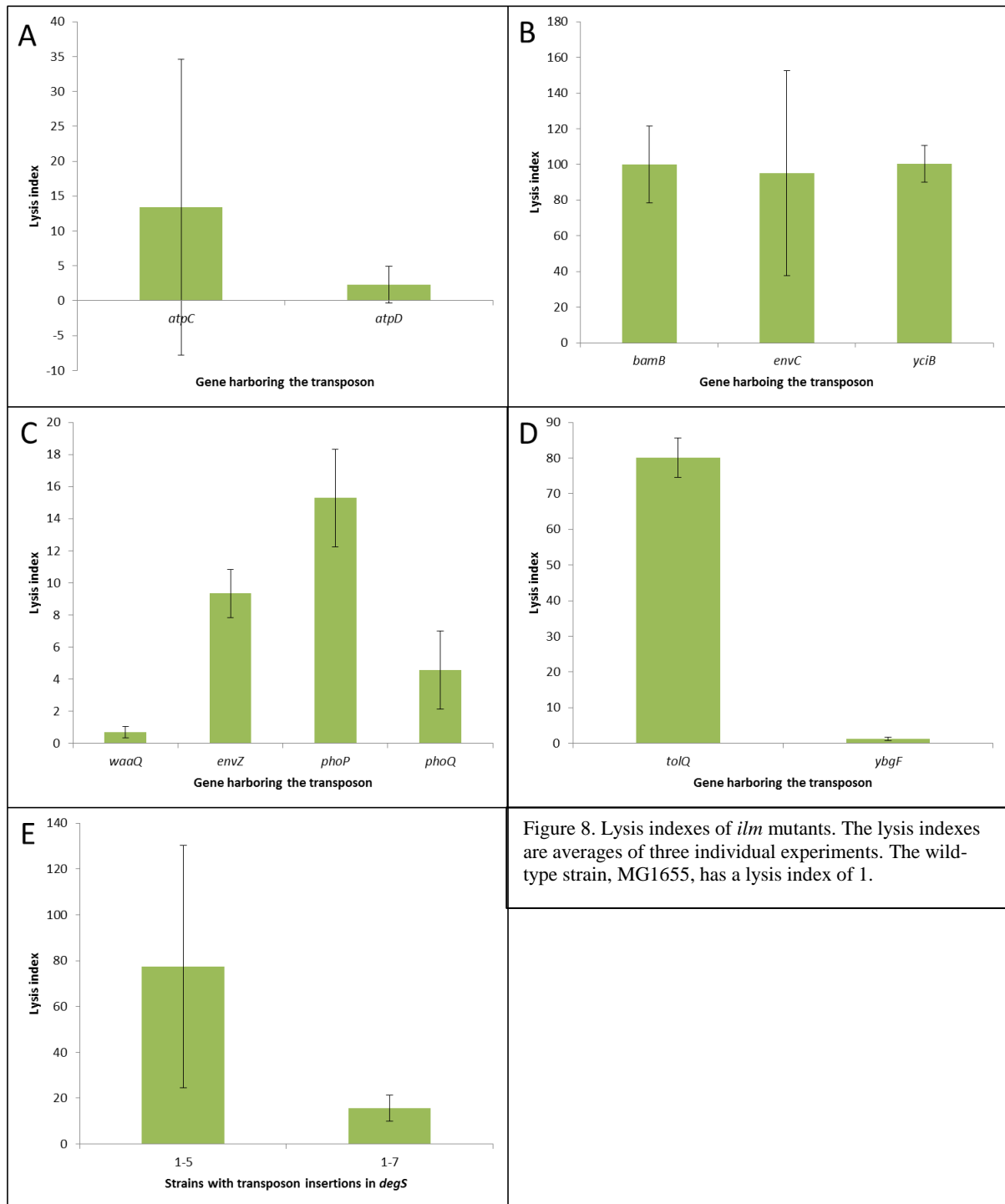
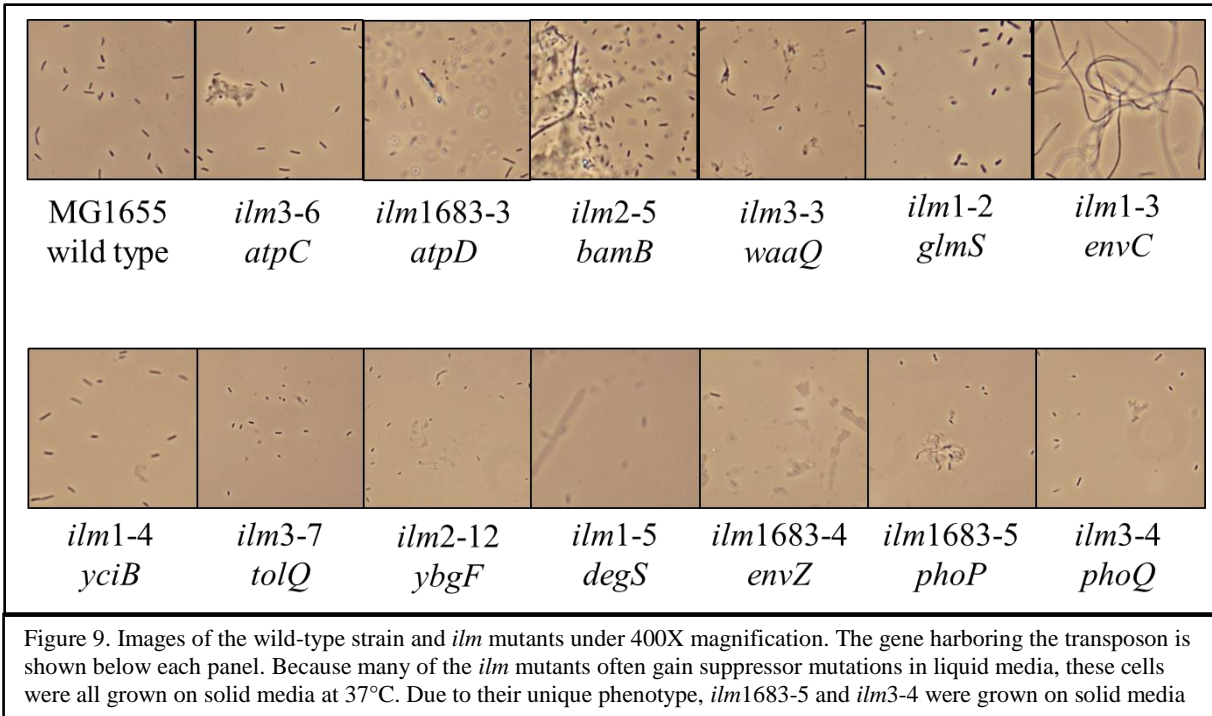


Figure 8. Lysis indexes of *ilm* mutants. The lysis indexes are averages of three individual experiments. The wild-type strain, MG1655, has a lysis index of 1.



### ***ilm* mutants with pleiotropic defects**

#### *Transposon insertions in atpC and atpD*

Three increased lysis mutants, *ilm3-6*, *ilm1683-3*, and *ilm1683-8*, have transposon insertions in genes that encode subunits of the ATP synthase machinery. In *ilm3-6*, the transposon insertion is in the gene *atpC* (Fig. 6a; Table 1), which encodes for the  $\epsilon$  subunit of ATP synthase (Futai et al., 1989). In *ilm1683-3* and *ilm1683-8*, the transposon insertion is in the gene *atpD* (Fig. 6a; table 1), which encodes for the  $\beta$  subunit of ATP synthase (Futai et al., 1989). *ilm3-6*, *ilm1683-3*, and *ilm1683-8* display the increased lysis mutation on solid media (Fig. 7b). In liquid culture, however, their lysis is highly variable, likely due to varying suppressor mutations (lysis indexes of  $13.382 \pm 21.178$  and  $2.317 \pm 2.624$  for strains with transposon insertions in *atpC* and *atpD*, respectively; Fig. 8a). Under 400X magnification, the living cells

resemble wild-type cells, but many of the cells have lysed resulting in a large amount of cellular debris (Fig. 9).

ATP synthase is a protein complex that synthesizes ATP from ADP and phosphate using an electrochemical gradient for energy (Futai et al., 1989). ATP is the main source of energy for many reactions within a cell, including numerous processes occurring within the cell envelope such as LPS transport (Ruiz et al., 2009) and lipoprotein translocation across the IM (Narita, 2011). Mutations that disrupt the normal functioning of ATP synthase, therefore, result in varied phenotypes due to the lowered levels of ATP. The transposon insertions in *atpC* and *atpD*, therefore, cause a pleiotropic phenotype which includes the increased lysis phenotype.

### ***ilm* mutants with defects in OM biogenesis**

#### *Transposon insertion in bamB*

One increased lysis mutant, *ilm2-5*, has a transposon insertion in the gene *bamB* (Fig. 6b; Table 1). *ilm2-5* displays the increased lysis phenotype both on solid media and in liquid media (Fig. 7b; lysis index of  $99.935 \pm 21.579$ ; Fig. 8b). Under 400X magnification, the living cells resemble wild-type cells, but many of the cells have lysed resulting in a large amount of cellular debris (Fig.9).

BamB is an OM lipoprotein component of the BAM complex, a multi-protein complex that assembles  $\beta$ -barrel proteins at the OM (Hagan et al., 2011; Knowles et al., 2009). Though viable,  $\Delta bamB$  strains have increased outer membrane permeability because of their defective OM biogenesis (Ruiz et al., 2005). Our results suggest that defects in OM biogenesis caused by the loss of BamB function also lead to an increase in cell lysis. Although BamB has already been characterized, the identification of *bamB* validates our screen because BamB is an OM-

biogenesis factor. In fact, our screen was validated through the identification of several mutants described below.

#### *Transposon insertion in waaQ*

One increased lysis mutant, *ilm3-3*, has a transposon insertion in *waaQ* (Fig. 6c; Table 1). Though *ilm3-3* does display the increased lysis mutation on solid media (Fig. 7b), it does not release more of its cytoplasmic contents in liquid media than the wild-type strain (lysis index of  $0.677 \pm 0.350$ ; Fig. 8c). Under 400X magnification, the living cells grown on solid media resemble wild-type cells, but many of the cells have lysed resulting in a large amount of cellular debris (Fig. 9).

WaaQ is involved in LPS biosynthesis. The inner core of LPS in *E. coli* contains three L-glycero-D-manno-heptose sugars, Hep I, Hep II, and Hep III (Yethon et al., 1998). WaaQ is a transferase that attaches Hep III to Hep II (Raetz and Whitfield, 2002). Additionally, WaaY, a protein that phosphorylates Hep II, cannot perform its enzymatic activity if WaaQ has not already attached Hep III to Hep II (Raetz and Whitfield, 2002). *ilm3-3*, therefore, is phenotypically similar to a strain lacking both WaaQ and WaaY. The increased lysis phenotype in *ilm3-3* is due to the halted biosynthesis of the inner core of this strain's LPS. Mutants defective in the synthesis of LPS core have been reported to have OM defects that lead to increased OM permeability (Nikaido, 2003). Therefore, our results suggest that biosynthesis of the entire LPS core is more important on solid medium than in liquid medium.

Together, identification of *bamB* and *waaQ* reveals that even mild defects in OM biogenesis weaken the integrity of the cell.

#### ***ilm* mutants with defects in peptidoglycan degradation or biosynthesis**

##### *Transposon insertions in glmS*

One increased lysis mutant, *ilm1-2*, has a transposon insertion in the gene *glmS* (Fig. 6d; Table 1). Three other increased lysis mutants, *ilm1-6*, *ilm2-1* and *ilm2-2*, were also identified as  $\Delta glmS$  strains because of their phenotypic similarities to *ilm1-2* and  $\Delta glmS::kan$  strain from the Keio Collection. All of these strains do not grow on minimal media and grow poorly on LB media, on which they display the increased lysis phenotype (Fig. 7a). They do, however, grow like the wild-type strain on LB media supplemented with D-glucosamine. These strains also display the increased lysis phenotype in liquid media (lysis index of  $789.020 \pm 228.923$ ). Under 400X magnification, the cells are thicker and rounder than wild type (Fig. 9).

GlmS is a glucosamine-6-phosphate synthase that is involved in the synthesis of UDP-N-acetyl glucosamine (UDP-GlcNAc), a precursor used in the biosynthesis of peptidoglycan and lipid A (Barreteau et al., 2008; Raetz and Whitfield, 2002). Specifically, GlmS converts D-fructose-6-phosphate to D-glucosamine-6-phosphate, the first step in the UDP-GlcNAc synthesis pathway (Barreteau et al., 2008). Because UDP-GlcNAc is required for both the peptidoglycan layer and the outer membrane, GlmS is an important protein in maintaining envelope integrity. Cells without suppressor mutations are able to survive without functioning GlmS only if D-glucosamine is added to the medium (Vogler et al., 1989). LB must contain some D-glucosamine but not enough for wild-type growth, resulting in small colonies and the increased lysis phenotype observed in our screen. Adding D-glucosamine to the LB, however, is adequate to support wild-type growth of these *ilm* mutants. This occurs because *E. coli* has a redundant pathway for glucosamine-6-phosphate synthesis involving D-glucosamine. NagE, an inner membrane protein, can transport D-glucosamine to the cytoplasm while simultaneously phosphorylating it (Park and Uehara, 2008). The fact that GlmS is important in the synthesis of both LPS and peptidoglycan explains why *glmS* mutants exhibit the strongest lysis phenotype.

## ***ilm* mutants with defects in septation or OM invagination**

### *Transposon insertions in envC*

Two increased lysis mutants, *ilm1-3*, *ilm1-8*, and *ilm3-2* have transposon insertions in *envC* (Fig. 6e; Table 1). *ilm3-2* was identified as a  $\Delta envC$  strain because of its phenotypic similarity to *ilm1-3*, *ilm1-8*, and the Keio Collection  $\Delta envC::kan$  strain and through amplification of the *envC* region in this strain. These strains display the increased lysis phenotype both on solid media and in liquid media (Fig. 7c; lysis index of  $85.961 \pm 57.529$ ; Fig. 8b), and under 400X magnification, long chains of cells with shared septa are visible (Fig. 9).

EnvC is a periplasmic protein involved in separation of daughter cells during cell division. It localizes to the divisome where it activates the amidases AmiA and AmiB. These amidases degrade portions of the peptidoglycan at the septum, allowing the peptidoglycan to split between the two daughter cells (Uehara et al., 2010). Cells without properly functioning EnvC, therefore, resemble cells without AmiA and AmiB, and the cells have difficulty separating during division, resulting in the long chains of cells visible under the microscope.

The gene *envC* is located in an operon with *yibQ* (Fig. 6e; Riley et al., 2006). These two genes are separated by only four base pairs, and *yibQ* does not have its own ribosomal binding site (Riley et al., 2006). These genes are, therefore, most likely translated together. Unlike EnvC, however, the function of the periplasmic protein YibQ is not understood (Misra et al., 2005). We tested whether the loss of YibQ function could also lead to the *Ilm* phenotype. We found that  $\Delta yibQ$  strains do not display the *Ilm* phenotype and are largely indistinguishable from wild type. Furthermore,  $\Delta yibQ\Delta amiA\Delta amiB$  strains resemble  $\Delta envC$  and  $\Delta amiA\Delta amiB$  strains. We also showed that overexpression of *yibQ* by controlling the expression of the gene through the

arabinose promoter does not result in any discernible phenotype. Therefore, we were unable to demonstrate that YibQ functions in the EnvC pathway.

#### *Transposon insertion in yciB*

One increased lysis mutant, *ilm1-4*, has a transposon insertion in *yciB* (Fig. 6f; Table 1). *ilm1-4* displays the increased lysis phenotype both on solid media and in liquid media (Fig. 7c; lysis index of  $100.311 \pm 10.199$ ; Fig. 8b). Under 400X magnification, the living cells resemble wild-type cells, but some of the cells have lysed resulting in cellular debris (Fig. 9).

YciB is an IM protein that is most likely involved in septation, but its exact function is not understood (Niba et al., 2007). *Shigella flexneri* strains lacking YciB filament inside host cells, although this phenotype has yet to be replicated in *S. flexneri* outside of host cells or in *E. coli* strains (Mac Siomoin et al., 1996). Additionally, *E. coli*  $\Delta yciB$  strains are less able to form biofilms (Niba et al., 2007).

We also confirmed that our transposon mutant behaves like a  $\Delta yciB::kan$  mutant. We then searched for additional phenotypes conferred by the  $\Delta yciB::kan$  allele and found that it also increases sensitivity to copper. We do not know whether this is related to the oxidizing properties of copper. The  $\Delta yciB::kan$  strain was consistently slightly more sensitive to ethylenediaminetetraacetic acid (EDTA), and EDTA and sodium dodecyl sulfate (SDS) than wild type (Table 2), suggesting mild OM defects (Ruiz et al., 2009). We also investigated whether we could find phenotypes caused by *yciB* overexpression. However, overexpression of *yciB* by controlling the expression of the gene through the arabinose promoter does not result in a phenotypic difference from the wild-type strain.

<b>Table 2. Minimum inhibitory concentrations (MICs) of the wild-type strain (MG1655) and the <math>\Delta yciB</math> strain.</b>			
	Cu <sup>2+</sup>	EDTA	EDTA with 0.5% SDS



MG1655	2.5 mM	25 mM	0.10 mM
MG1655 $\Delta$ <i>yciB::kan</i>	0.6 mM	12.5 mM	0.05 mM

#### *Transposon insertions in tolQ and ybgF*

One increased lysis mutant, *ilm3-7*, has a transposon insertion in *tolQ* (Fig. 6g; Table 1), and another increased lysis mutant, *ilm2-12*, has a transposon insertion in *ybgF* (Fig. 6g; Table 1). These strains both display the increased lysis phenotype both on solid media (Fig. 7c). In liquid media, *ilm3-7* also displays the increased lysis phenotype, but *ilm2-12* does not release more of its cytoplasmic contents than the wild-type strain (lysis indexes of  $80.148 \pm 5.532$  and  $1.263 \pm 0.471$  for strains with transposon insertions in *tolQ* and *ybgF*, respectively; Fig. 8d). Under 400X magnification, the living cells are smaller and rounder than wild type, and some of the cells have lysed resulting in cellular debris (Fig. 9).

Both TolQ and YbgF are protein components of the Tol-Pal complex which spans the cell envelope from the IM to the OM. It has been shown that Tol-Pal proteins localize to the divisome during cell division, and the complex is involved in the invagination of the OM during separation (Gerding et al., 2007). Strains lacking functional TolQ have decreased cellular integrity and have been shown to release OM vesicles (Bernadac et al., 1998). If any portion of the Tol-Pal complex is missing, the function of the complex could be compromised. *ilm3-7* and *ilm2-12*, therefore, display the increased lysis phenotype because their Tol-Pal complexes are unable to function completely. Together, the *envC*, *yciB*, *tolQ*, and *ybgF* mutants described in this section reveals that defects in cell separation caused by defects in septal peptidoglycan or OM invagination compromise cell integrity.

#### ***ilm* mutants with defects in regulation of cell envelope stress responses**

##### *Transposon insertions in degS*

Two increased lysis mutants, *ilm1-5* and *ilm1-7*, have transposon insertions in *degS* (Fig. 6h; Table 1). These strains display the increased lysis phenotype both on solid media and in liquid media, but the increased lysis phenotype of *ilm1-5* in liquid media is much more pronounced than the phenotype of *ilm1-7* (see below) (Fig. 7a; lysis indexes of  $77.375 \pm 52.797$  and  $15.658 \pm 5.574$  for *ilm1-5* and *ilm1-7*, respectively; Fig. 8e). Under 400X magnification, the living cells are smaller than wild-type cells, and many of the cells have lysed resulting in a large amount of cellular debris (Fig. 9).

DegS is an IM protein involved in the  $\sigma^E$  stress response (Ades, 2008). Under normal conditions, the PDZ domain of DegS inhibits its function. Misfolded OM porins within the periplasm, a sign of  $\beta$ -barrel assembly defects, causes a conformational change in DegS that moves the PDZ domain to expose the catalytic domain. DegS then cleaves the periplasmic end of RseA, an IM protein that sequesters  $\sigma^E$  at the IM.  $\sigma^E$  is an alternative sigma factor that allows for the transcription of genes encoding proteins that aid OM protein folding. After DegS cleaves the periplasmic portion of RseA, RseB, a periplasmic protein that enhances RseA's function, is released; RseP cleaves the transmembrane portion of RseA; ClpXP cleaves the cytoplasmic portion of RseA; and finally  $\sigma^E$  is released so that it can function as a sigma factor (Ades, 2008).

DegS is an essential protein (Alba et al., 2001); a cell lacking DegS would be unable to release  $\sigma^E$  and would, therefore, lack this important stress response. *ilm1-5* and *ilm1-7*, however, are viable. We hypothesized that perhaps the insertions are gain of function alleles that could interfere with the PDZ domain of DegS, resulting in a cell with an increased stress response. This cell would have a similar phenotype to a  $\Delta rseA$  strain, a strain that has been shown to display the increased lysis phenotype because of the increased levels of active  $\sigma^E$  (Kabir et al., 2005; Nitta et al., 2000). Alternatively, the mutants might have decreased  $\sigma^E$  activity if these are partial loss of

function alleles. To test this, we determined DegP levels in the mutant because *degP* transcription is upregulated by  $\sigma^E$ . We found that levels of DegP are lower in the mutants than wild type, suggesting that the  $\sigma^E$  stress response is decreased in *ilm1-5* and *ilm1-7*. This suggests that both transposon alleles are partial loss of function alleles. Further research is needed to determine how the transposon insertions in *ilm1-5* and *ilm1-7* are affecting the cells. These strains, however, easily gain suppressor mutations in culture, making further study difficult. In fact, we believe, that the differences in the lysis indexes of *ilm1-5* and *ilm1-7* could be caused by the presence of suppressors in *ilm1-7*.

#### *Transposon insertion in envZ*

One increased lysis mutant, *ilm1683-4*, has a transposon insertion in *envZ* (Fig. 6i; Table 1). *ilm1683-4* displays the increased lysis phenotype both on LB plates and in overnight culture (Fig. 7a; lysis index of  $9.344 \pm 1.499$ ; Fig. 8c). Under 400X magnification, the living cells generally resemble wild-type cells though some cells are very large and deformed, and many of the cells have lysed resulting in a large amount of cellular debris (Fig. 9).

EnvZ is part of a two-component system with EnvZ acting as the sensor kinase/phosphatase and OmpR acting as the response regulator. EnvZ senses the osmolarity of the surrounding medium and then transmits the signal to OmpR; OmpR then controls the expression of two abundant OM  $\beta$ -barrel porins, OmpF and OmpC. OmpF is larger and predominates at low osmotic pressure. OmpC is smaller and predominates at high osmotic pressure (Stock et al., 1990). Because these two porins play a major role in OM permeability and decreasing the concentration of porins in the outer membrane leads to increased OM permeability (Nikaido and Vaara, 1985), cell integrity defects in *ilm1683-4* are likely the result of misregulation of these porins.

### *Transposon insertions in phoP and phoQ*

One increased lysis mutant, *ilm1683-5*, has a transposon insertion in *phoP* (Fig. 6j; Table 1), and another increased lysis mutant, *ilm3-4*, has a transposon insertion in *phoQ* (Fig. 6j; Table 1). These strains both display the increased lysis phenotype both in liquid media, though the lysis of *ilm1683-5* is more pronounced than the lysis of *ilm3-4* (lysis indexes of  $15.283 \pm 3.049$  and  $4.562 \pm 2.446$  for strains with transposon insertions in *phoP* and *phoQ*, respectively; Fig. 8c). These strains display the increased lysis phenotype on solid media only when kanamycin is present and the strains are grown at 42°C, and the strains gain many suppressor mutations under these conditions (Fig. 7d). Under 400X magnification, the living cells generally resemble wild-type cells for both strains, but many of some the cells have lysed resulting in a cellular debris (Fig. 9).

Together, PhoP and PhoQ form a two component system with PhoQ acting as the histidine kinase and PhoP acting as the response regulator. The sensor PhoQ is stimulated by low pH (Prost et al., 2007) and reduced proteins in the periplasm (Lippa and Goulian, 2012), and it is repressed by high concentrations of  $Mg^{2+}$  (Eguchi and Utsumi, 2008). PhoP controls the transcription of many genes, including genes whose products are involved in  $Mg^{2+}$  transport and LPS modification (Groisman, 2001).

The presence of the increased lysis phenotypes of *ilm1683-5* and *ilm3-4* on solid media that contains kanamycin and the absence on solid media that does not contain kanamycin indicates increased sensitivity to kanamycin in these strains. We have not yet determined if this sensitivity is specific for this antibiotic. For example, it is possible that sensitivity is increased because cells are more permeable to kanamycin and other antibiotics. Antibiotic specificity can be determined by testing resistance to other antibiotics to which *E. coli* is naturally resistant or

by introducing a gene conferring resistance to an antibiotic different than kanamycin. If the increased lysis phenotype persists in the presence of these antibiotics, then the phenotype is not antibiotic specific.

If the phenotype does not persist, it will be interesting to investigate why these mutants are more sensitive to kanamycin. A possibility is that the presence of the kanamycin cassette at the *phoPQ* locus increases sensitivity because of possible alterations on the expression of the downstream gene *ycfD* which encodes an protein of unknown function (Fig.6j).

Location specificity can be tested using the  $\Delta phoP$  and  $\Delta phoQ$  alleles from the Keio Collection. After introducing each of the alleles into wild type, their respective kanamycin cassettes can be excised using the Flp recombinase (Cherepanov and Wackernagel, 1995). New kanamycin resistance genes could be introduced into the resulting  $\Delta phoP$  and  $\Delta phoQ$  strains. If the increased lysis phenotype persists in these strains that have the kanamycin resistance in a different location, then the phenotype is not location specific. In this situation, the lack of PhoP or PhoQ is the cause of the phenotype. If the phenotype does not persist, then the kanamycin resistance gene's location on the chromosome is the cause of the phenotype, not the lack of PhoP or PhoQ. Additional research such as this is needed to elucidate the specificity and exact nature of the increased sensitivity to kanamycin in *ilm1683-5* and *ilm3-4*.

## Conclusions

The screen successfully identified factors involved in *E. coli* cell envelope integrity. The majority of proteins identified by this screen were involved in biogenesis of the OM, synthesis of the peptidoglycan layer, processes that are required for proper cell separation, and envelope stress responses. The screen, therefore, is an incredibly useful tool in identifying proteins involved in various aspects of cell envelope biogenesis. Although the majority of the proteins

that were identified in *E. coli* have already been characterized, the screen was not saturated. Therefore, it is possible that many other factors involved in envelope integrity could be identified through more trials of this screen.

Additionally, this screen is not restricted to only *E. coli*. The EZ-Tn5 Transposome can mutate numerous species, including Gram-negative bacteria such as *Cronobacter sakazakii* and *Proteus vulgaris* (Goryshin et al., 2000; Hartmann et al., 2010), Gram-positive bacteria such as *Clostridium perfringens* and *Rhodococcus equi* (Miranda-Casoluengo et al., 2005; Vidal et al., 2009), and even the eukaryote *Saccharomyces cerevisiae* (Goryshin et al., 2000). Furthermore, *lacZ* can be expressed in various species (Ghim et al., 2010), and the screen can be even modified for other cytoplasmic enzymes with colorimetric substrates. Additionally, the screen is fast and inexpensive, allowing multiple trials to be performed easily. This screen, therefore, can be a valuable tool in researching cell envelopes of various microbial species.

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